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SUBJECT: Annual Report for Award Number DAMD17-02-1-0159

"Hepatocyte Growth Factor and Interleukin-6 in Prostate Cancer Bone Metastasis"

Overview of tasks accomplished in the first year. In the first year of the award, we evaluated the design and progression of experiments of the project. We completed the first task, listed in the statement of work. In addition, I moved from Cornell University to the Fred Hutchinson Cancer Research Center. The environment at FHCRC is conducive to abroad genome wide analysis to determine the entire spectrum of androgen-regulated growth factors in bone, which is listed under task 4. As a first step, we analyzed the expression of hepatocyte growth factor (HGF) and interleukin-6 (IL-6) in mouse bone, which is part of task 1. We also evaluated changes in expression levels as a result of androgen deprivation of bone and bone marrow. The assay development and results are listed in detail in the following section.

- **Development of assays for completion of task 1:**
  1. Isolation of RNA from mouse bone. Isolation of undegraded RNA proved to be difficult. We circumwent this problem by generating a fine bone powder in a dry ice bath prior to extraction with TRIZOL®.

  2. Semi-quantitative RT-PCR for measurements of HGF, IL-6, Cbfa-1, osteocalcin, osteonectin and actin. After determination of the quality of RNA we determined RNA input for the reverse transcriptase reaction, which is the first step in the RT-PCR. 2 ug of input RNA was in the linear range and found to be the optimal amount of input RNA. 2 ug of RNA were used in all experiments. We tested several primer pairs to find the best for PCR. The PCR fragments for each primer pair were between 300 and 500 bp. Whenever possible, we included a reference primer pair in the PCR, which allowed standardization of each PCR run. However, this was not possible for the HGF PCR, since we encountered primer interference with several reference primers. Therefore, HGF m-RNA expression was measured in a separate PCR-tube and compared to reference primer sets in parallel tubes under identical PCR.

For each primer set, we determined the linear range (shown in figure 1B).

  3. Radioactive quantification of PCR products. To obtain better and more direct quantification, we attempted to label PCR products with ^{32}P-ATP. However, these experiments did not provide the anticipated improvement.

  4. Measurements of expression of the Met receptor by Western blotting. For Western blot analysis, powdered bone was solubilized in RIPA buffer and 100ug protein loaded per lane.

- **Results for completion of task 1:**

Changes in bone maturation upon orchiectomy in mice. Since the absence of androgen effects the integrity of bone, we measured m-RNA expression of genes that are expressed in a sequential order during bone development. These first included Cbfa-1 and osteonectin. We reasoned that the Cbfa-1 to

osteonectin ratio indicates the maturity of bone, high Cbfa-1 and low osteonectin representing immature bone.

Measurements of HGF/SF in normal and orchiectomized mice

To measure HGF m-RNA expression, bone and bone marrow were separated. While bone marrow contains immature osteoblastic cells, the bone harbors mature mature osteoblasts and osteocytes. Figure 1 shows, that osteonectin m-RNA is decreased in orchiectomized bone. This is consistent with features of osteoporosis that we saw histologically in the bone of mice that were orchictomized 1 year prior to analysis. Since it is not certain which cells make HGF/SF, we compared bone and bone marrow from the mice.

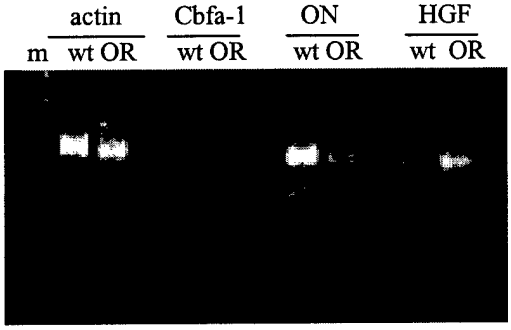


Figure 1: RT-PCR with RNA from bone marrow stromal cell. Bone marrow was separated from bone prior to preparing RNA. Primers to evaluate m-RNAs of HGF, osteonectin (ON), actin and Cbfa-1 were used in the same PCR reaction. Bands were quantified densitometrically to calculate relative expression of HGF.

This study shows that (1) osteonectin m-RNA is decreased in the bone marrow of androgen deprived mice, suggesting that there are fewer osteoblastic cells. In contrast HGF is increased. However, we don't know whether HGF is only synthesized by mesenchymal cells. It is possible that lymphocytes also make HGF and andrgen depletion leads to a lymphocytosis, which could account for the increased HGF production (Olsen et al., 2001).

Analyzing HGF m-RNA synthesis in bone did not show any differences between orchiectomized and normal bone.

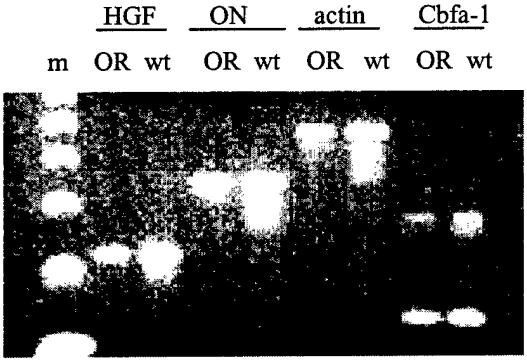
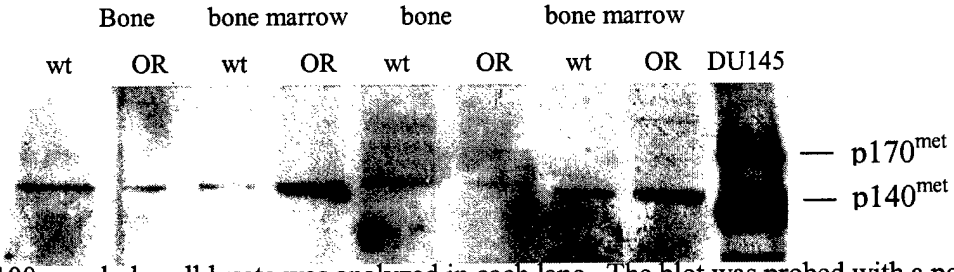


Figure 2: RT-PCR with RNA from bone of orchiectomized and wild type mice. Primers to evaluate m-RNAs of HGF, osteonectin (ON), actin and Cbfa-1 were used in the same PCR reaction. Bands were quantified densitometrically to calculate relative expression of HGF.

These experiments show that the effects of androgen withdrawl are most pronounced on mesenchymal bone marrow cells and we will use the bone marrow preparation in addition to bone in subsequent studies.

Measurements of the Met receptor in bone and bone marrow. Expression of Met was analyzed by Western blot (Figure 3).



100 ug whole cell lysate was analyzed in each lane. The blot was probed with a polyclonal anti-met (Santa Cruz).

The HGF receptor, met, is highly expressed in the bone marrow of androgen deprived mice, but not in bone. This is consistent with our results that the bone marrow is most effected by androgen deprivation.

Measurement of IL-6 in normal and orchiectomized mice. In contrast to published reports that clearly show the modulation of IL-6 expression by steroid hormones in bone cells (Gornstein et al., 1999; Hierl et al., 1998; Hofbauer et al., 1999; Hurley et al., 1996; Linkhart et al., 1991; Manolagas, 1998; Manolagas et al., 1995; Navarro et al., 1991; Rifas et al., 1995; Ritchie et al., 1997; Sandhu et al., 1999; Swolin-Eide and Ohlsson, 1998; Zhang et al., 1998), we were unable to detect changes of IL-6 at the m-RNA expression level. It is possible, that the C6/BL57 strain is less sensitive the androgenic regulation of IL-6. Furthermore, we did not detect changes in HGF or met expression in the IL-6 knockout versus normal mice.

Therefore we decided to analyze the global changes of gene expression as they occur upon androgen deprivation in mouse bone. Because of the strong c-DNA array community at FHCRC, we expect tat this project will be successful in identifying growth factors that are increased upon androgen ablation. As a next step these can be tested directly in human specimens of prostate cancer metastasis, in a newly established resource at the University of Washington, directed by Dr. Bob Vessella.

During the last year, I moved from Cornell Medical College to the Fred Hutchinson Cancer Research Center. This move has slowed the research during the first year of the award. However, the lab is fully operating at this point and I do not anticipate any problems for the rest of the funding period.

Probable effects of moving the project:

The proposed project has a much greater chance of success at the Fred Hutchinson Cancer Research Center, than at Cornell. Core facilities are more advanced, especially the c-DNA array core. There is another investigator, an associate member of the institute who's laboratory is mainly interested in androgen-regulated gene expression and has identified several androgen regulated genes in the LNCaP cells. This group has also developed the bioinformatics and databases for categorizing the genes from their gene discovery projects.

The c-DNA core facility at Fred Hutchinson has a homemade mouse c-DNA array, which is much cheaper than the commercial array. They also provide a full service of labeling probes, hybridization and data analysis. It will be faster to identify androgen regulated genes in mouse bone, with the expertise of a well established c-DNA array facility.

With the help of the Veterinarian at Fred Hutchinson, I have identified a vendor who can supply castrated mice, thus accelerating the procurement of bone from androgen-deficient mice.

I have recruited a new post-doctoral fellow for the project who will take Dr. Xueke You's place. Chang Xu has spend the last year in a c-DNA facility at the University of Washington and also has a Masters in Biostatitics. He has the perfect technical background for the proposed project and Will rapidly be getting data using the c-DNA approach to identify androgen regulated growth factors and growth factor receptors in bone.

Undoubtedly, the Fred Hutchinson Cancer Research Center will provide a beneficial intellectual and technical environ to achieve the goals of the proposed project.

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